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Effect of Lipase Immobilization on Resolution of (*R, S*)-2-Octanol in Nonaqueous Media Using Modified Ultrastable-Y Molecular Sieve as Support

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Abstract

The lipase from *Penicillium expansum* PED-03 (PEL) was immobilized onto modified ultrastable-Y (USY) molecular sieve and the resolution of (*R, S*)-2-octanol was carried out in a bioreactor in nonaqueous media by the immobilized lipase. It was found that the conversion rate, enantiomeric excess (*ee*) value, and enantioselectivity (*E*) value of the resolution catalyzed by PEL immobilized on modified USY molecular sieve were much higher than those of the reaction catalyzed by free PEL and PEL immobilized on other supports. Immobilized on modified USY molecular sieve, the PEL exhibited obvious activity within a wider pH range and at a much higher temperature and showed a markedly enhanced stability against thermal inactivation, by which the suitable pH of the buffer used for immobilization could be "memorized." The conversion rate of the reaction catalyzed by PEL immobilized on modified USY molecular sieve reached 48.84%, with excellent enantioselectivity (average *E* value of eight batches >460) in nonaqueous media at "memorial" pH 9.5, 50°C for 24 h, demonstrating a good application potential in the production of optically pure (*R, S*)-2-octanol.

Index Entries: Modified ultrastable-Y molecular sieve; immobilized lipase; resolution; 2-octanol; catalytic property.

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Introduction

Chiral 2-octanol is one of the most important building blocks for the preparation of liquid crystal materials, as well as an important intermediate of many optically active pharmaceuticals. Interest in the enzymatic resolution of (*R, S*)-2-octanol has increased markedly owing to the rapid progress in the technique of enzyme-catalyzed reaction in nonaqueous media in the last two decades. Kinetic resolution of (*R, S*)-2-octanol by enantioselective lipase has been reported in the literature *(1–3)*. However, these reports mainly focus on the selection of free lipase and optimization of reaction conditions. Few studies are exclusively directed toward lipase immobilization on molecular sieve or related materials and the effect of immobilization on conversion, enantiomeric excess (*ee*), and enantioselectivity (*E*).

Recently, we have isolated a strain named *Penicillium expansum* PED-03 from a rap oil manufacturer in China and found that this strain could produce an alkaline lipase (PEL) at high level *(4)* with fine enantioselectivity in the resolution of (*R, S*)-2-octanol. Because free lipase is sensitive to the water in nonaqueous media and easy to agglomerate, which has a negative influence on the resolution reaction, it is necessary for the lipase to be immobilized on a certain support. In the present study, we found that modified ultrastable-Y (USY) molecular sieve was very suitable for use as support for the immobilization of PEL, and that the USY molecular sieve not only could immobilize the lipase commendably, but also could maintain the required microenvironment for the resolution reaction catalyzed by PEL in nonaqueous media. The enantioselectivity and the activity of PEL immobilized on modified USY molecular sieve were much higher than those of free PEL and PEL immobilized on other supports in the resolution of (*R, S*)-2-octanol through transesterification in nonaqueous media with a stable catalytic performance.

In this article, we describe a new method to immobilize PEL using modified USY molecular sieve as support and its evaluation for the biocatalytic resolution of (*R, S*)-2-octanol in microaqueous media.

Materials and Methods

Enzyme and Reagents

Lipase powder (80,000 U/g) was produced in submerged fermentation by *P. expansum* PED-03 in our laboratory *(4)*. The enzyme was used for enzymatic reaction without further purification after lyophilization.

(*S*)-2-Octanol, (*R*)-2-octanol, and (*R, S*)-2-octanol were purchased from Shijiazhuang Jida Fine Chemicals (Hebei, China). USY molecular sieve was obtained from Wenzhou Huahua (Zhejiang, China). Other reagents used for the enzymatic resolution were of analytical grade and were obtained commercially. All nonaqueous solvents were dried with anhydrous Na_2SO_4 before being used for resolution.

Modification of Molecular Sieve

Ten grams of Al_2O_3 were added as binder to 50 g of USY molecular sieve in a 100-mL beaker. After agitating the mixture completely, it was kneaded and extruded and then dried at room temperature. The dried mixture was baked in a muffle furnace at 600°C for 3 h and was ground to pass a 40-mesh sieve to be used as modified USY molecular sieve to immobilize PEL.

Immobilization of PEL

Immobilization of PEL on Modified USY Molecular Sieve

One gram of PEL was dissolved in 50 mL of 0.1 *M* Na_2CO_3-$NaHCO_3$ buffer (pH 9.5) in a 250-mL Erlenmeyer flask. Then 30 g of modified USY molecular sieve were added to the PEL solution, and the flask was put into a 25°C homeothermia water bath shaker at 150 rpm for the adsorption process of PEL onto modified USY molecular sieve for 12 h. After the adsorption at equilibrium, the immobilized lipase on modified USY molecular sieve was collected by centrifugation (4000 rpm, 5 min), washed several times with the same buffer, dried at room temperature, and used as immobilized lipase for the resolution of (*R, S*)-2-octanol.

Immobilization of Lipase on Sodium Alginate

One hundred milligrams of PEL were dissolved in 3 mL of 0.1 *M* Na_2CO3-$NaHCO_3$ buffer (pH 9.5) and mixed with 3 mL of 2% sodium alginate. Then the mixture was magnetically stirred for 6 h. When it was homogenized, the mixture was injected into 0.1 *M* $CaCl_2$ with a small injector. As they hardened, the spherules were taken out and washed several times with the same buffer, then dried at room temperature and used as immobilized lipase for the resolution of (*R, S*)-2-octanol.

Immobilization of Lipase on Silica Gel, Macroporous Resin, and Diatomite

The method was the same as that of immobilization of PEL on modified USY molecular sieve.

Enzymatic Resolution

Resolution was performed in a bioreactor containing 5 mL of (*R, S*)-2-octanol, 5.8 mL of vinyl acetate, 50 mL of *n*-hexane, 10,000 U of immobilized lipase, and 0.8% water at 50°C for 24 h. The resolution process was monitored by high-performance liquid chromatography (HPLC), and the conversion rate (*c*), *ee*, and *E* are defined as follows *(5–7)*:

$$ee\ (\%) = \frac{[S-R]}{[S+R]} \times 100$$

$$c\ (\%) = \frac{ee_s}{ee_s + ee_p} \times 100$$

$$E = \frac{\ln[(1-c)\,(1-ee_s)]}{\ln[(1-c)\,(1+ee_s)]} = \frac{\ln[1-c\,(1+ee_p)]}{\ln[1-c\,(1-ee_p)]}$$

in which *S* and *R* are the concentrations of the (*S*)-enantiomer and (*R*)-enantiomer, respectively; and ee_s and ee_p are the *ee* value for the substrate and *ee* value for the product, respectively.

Analysis of Lipase Activity

The activity of free and immobilized lipase was determined according to a slightly modified olive oil emulsion method *(8,9)*, and all reactions were performed at 35°C unless otherwise indicated. The assay mixture contained 5 mL of olive oil, 4 mL of 0.2 *M* glycine-NaOH (pH 9.5), 0.2 mL of 1 *M* $CaCl_2$, and 200 mg of immobilized lipase. The reaction was stopped by the addition of 15 mL of 95% ethanol. Fatty acids released were determined by titration with potassium hydrogen phthalate standardized with 50 m*M* NaOH. One unit of lipase activity was defined as the amount of lipase that librated 1 μmol of fatty acid/min under the assay condition.

HPLC Analysis

The conversion rate, *ee*, and *E* were determined by HPLC according to a published method with a slight modification *(3)*. The chromatographic conditions were as follows: SYLTECH-YMC column (150 × 4.6 mm), ultraviolet detector, 220-nm detection wavelength, 1.0 mL/min flow rate, mobile phase of 0.01 m*M* H_3PO_4-CH_3CN (37:63), 20-μL injection volume.

Results and Discussion

Immobilization of PEL

It was reported that the support used for immobilization of lipase played an important role in the enzymatic reaction in nonaqueous media, and that the lipase activity could be much higher when immobilized on a suitable support *(10,11)*. The resolution of (*R, S*)-2-octanol by PEL immobilized on different supports was carried out to investigate the effect of support on the enzymatic reaction (Table 1). As shown in Table 1, the performance of PEL immobilized on modified USY molecular sieve was much better than that of free PEL and PEL immobilized on other supports. The conversion rate of the reaction catalyzed by PEL immobilized on modified USY molecular sieve reached 48.84%, which was 3.07 and 36.18 times higher than that of the reaction catalyzed by free PEL and PEL immobilized on sodium alginate lipase, respectively. In addition, the *E* value of the reaction catalyzed by PEL immobilized on modified USY molecular sieve reached 560.32, which was 52.81 and 42.58 times higher than that of the reaction catalyzed by free and sodium alginate–immobilized lipase, respectively.

USY molecular sieve is a type of mesoporous material that possesses a regular hexagonal array of uniform pore openings with a broad spectrum of possible pore diameters between 2 and 20 nm, depending on the template used during synthesis. The structural characteristics of USY molecu-

Table 1
Effect of Immobilization on Catalytic Properties of PEL in Nonaqueous Media[a]

Carrier	ee_S[b]	ee_P[b]	C (%)[b]	E[b]
Control[c]	15.19	80.28	15.91	10.61
USY molecular sieve	94.27	98.75	48.84	560.32
Diatomite	52.81	91.37	36.63	37.15
Silica gel	23.35	86.64	21.23	17.52
Macroporous resin	48.07	90.15	34.78	31.02
Sodium alginate	1.17	85.72	1.35	13.16

[a]PEL was immobilized onto different supports and all reactions were carried out at 50°C for 24 h.
[b]Determined by HPLC.
[c]Control, free lipase.

lar sieve are highly suitable for the immobilization of enzymes *(12)*. For example, the large pore size should allow the bulky enzyme molecules to diffuse into the pore. In addition, the terminal silanol groups present on the surface of USY molecular sieve should facilitate immobilization of enzymes via hydrogen bonding. Moreover, enclosure of the protein in a well-defined space may also help prevent denaturing of the protein and enhance enzyme stability. Furthermore, the water in the pores of USY molecular sieve should maintain the required microenvironment for the resolution catalyzed by lipase in nonaqueous media. Dumitriu et al. *(13)* reported that the catalytic activity of MCM-36-immobilized lipase was approximately two times higher than that of the free lipase in the acylation reaction of alcohols (1-butanol and 1-octanol) by vinyl esters (vinyl acetate and vinyl stearate). Salis et al. *(14)* found that immobilization on Accurel MP1004 improved lipase performance in terms of both activity and substrate conversion. Takahashi et al. *(15)* found improved stability and catalytic activity of horseradish peroxidase immobilized in ordered mesoporous silica materials in organic solvent. In the present work, we found that the conversion rate, ee, and E of the resolution of (*R, S*)-2-octanol catalyzed by PEL immobilized on modified USY molecular sieve were much higher than those of the resolution catalyzed by free lipase as well as lipase immobilized on other supports.

Effect of Immobilization on Characteristics of PEL

Optimum Temperature for Reaction

The resolution of (*R, S*)-2-octanol catalyzed by PEL immobilized on modified USY molecular sieve was carried out at different temperatures in nonaqueous media to investigate the effect of immobilization on the optimum reaction temperature of PEL. As shown in Fig. 1, the optimum temperature of immobilized PEL was 50°C, which was much higher than that of free lipase (35°C), and the immobilized PEL exhibited obvious activity within a wider temperature range compared with the free lipase.

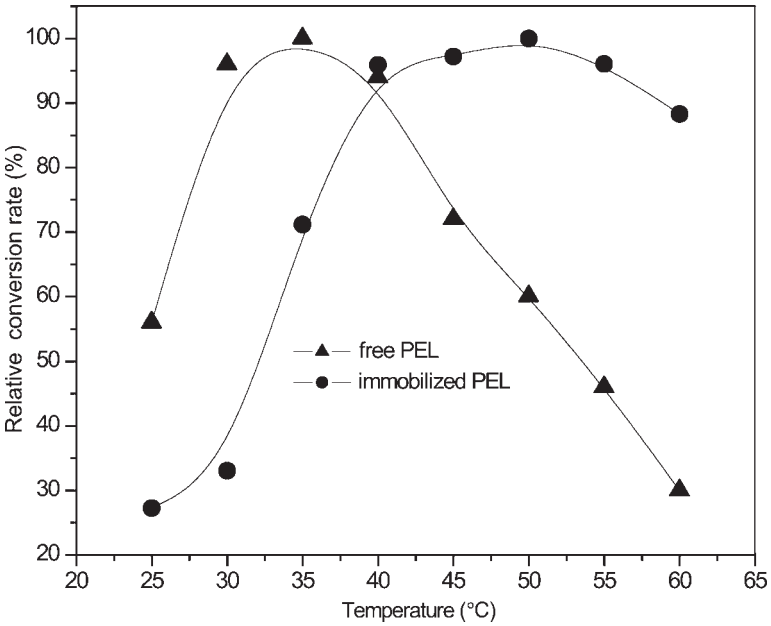

Fig. 1. Effect of immobilization on optimum temperature of PEL in nonaqueous media. PEL was immobilized onto modified USY molecular sieve and all reactions were carried out for 24 h at 25–60°C.

Immobilization of enzyme played an important role in the enzymatic reaction in nonaqueous media, and the effect of immobilization on the optimum reaction temperature differed a great deal according to different enzymes and supports *(16–18)*. The shift in suitable temperature for the resolution of (*R, S*)-2-octanol catalyzed by PEL immobilized on modified USY molecular sieve could be ascribed to the integrated effects of mass transfer, diffusion, and inactivation. Relative conversion rate of the reaction catalyzed by immobilized PEL was lower than by free PEL at a temperature less than 40°C, basically because of the mass transfer resistance. Owing to the easier transfer of substrates and diffusion of products at 40°C, this negative influence of mass transfer resistance could be counteracted completely and, thus, relative conversion rate of the reactions catalyzed by immobilized and free PEL were equal. However, when the temperature was higher than 50°C, relative conversion rate also decreased, because PEL was partly inactivated by the high temperature. Because the modified USY molecular sieve could maintain the preferable microenvironment, the relative conversion rate catalyzed by immobilized PEL decreased slower than by the free one.

Thermostability

After incubation at different temperatures in *n*-hexane for 6 h, the immobilized PEL was used as biocatalyst for the resolution of (*R, S*)-2-octanol to investigate the effect of immobilization on the thermostability of

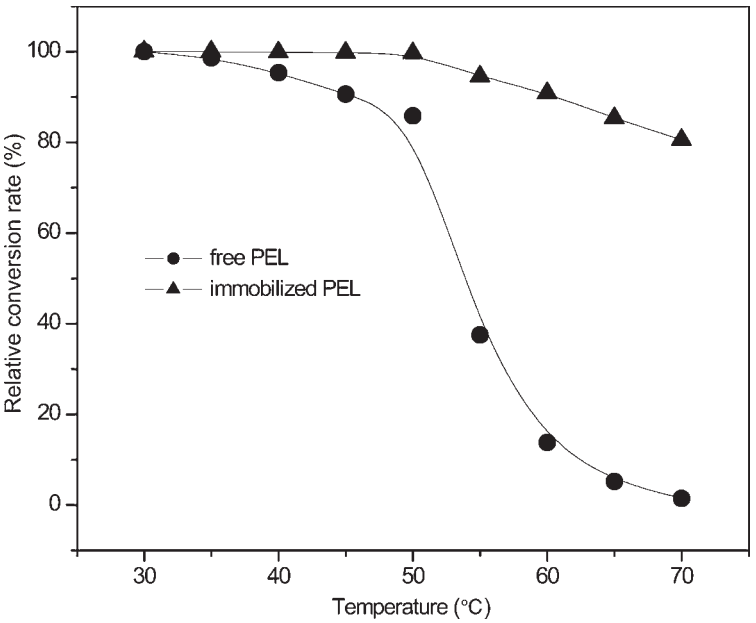

Fig. 2. Effect of immobilization on thermostability of free and immobilized PEL. PEL was immobilized onto modified USY molecular sieve and all reactions were carried out at 50°C for 24 h.

PEL (Fig. 2). It was found that the immobilized PEL was fairly stable when the temperature was lower than 50°C. Even at 70°C, the immobilized PEL still achieved more than 80% of the maximum activity. By contrast, the activity of free PEL decreased with an increase in temperature: it decreased sharply at a temperature higher than 50°C and was entirely inactivated when the temperature reached 70°C. This suggested that the thermostability of PEL was greatly improved when immobilized onto modified USY molecular sieve.

The thermostability of enzyme in nonaqueous media is related to the effect of the presence of a small amount of water on kinetic rigidity and thermodynamic stability, which are of great importance to the conformation of enzyme. Excessive rigidity will inactivate the enzyme in nonaqueous media, but too much flexibility will lead the conformation of PEL to a thermodynamic stable state, which also can inactivate the enzyme *(19)*. The moderate content of water in the pores of modified USY molecular sieve was able to maintain the "moist" microenvironment for PEL in the resolution of (*R, S*)-2-octanol in nonaqueous media, which was in favor of the moderate flexibility of PEL to enhance stability against thermal inactivation and led the immobilized PEL to reach a suitable and stable equilibrium point between kinetic rigidity and thermodynamic stability. The conformation of PEL was therefore stabilized and the performance of PEL was improved greatly.

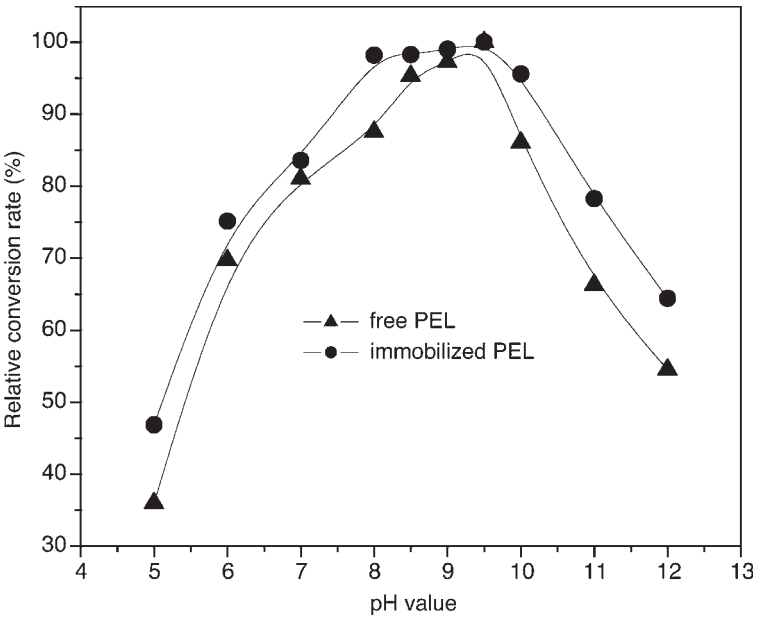

Fig. 3. Effect of immobilization on optimum reaction pH of PEL in nonaqueous media. PEL was immobilized onto modified USY molecular sieve in different pH buffers and reactions were carried out at 50°C for 24 h. The buffers used were as follows: pH 5.0–6.0, 0.1 *M* citrate; pH 7.0–8.0, 2 *M* NaH_2PO_4-Na_2HPO_4; pH 9.0–10.0, 0.2 *M* glycine-NaOH; pH 11.0–12.0, 0.05 *M* Na_2HPO_4-0.1 *M* NaOH.

Optimum pH for Reaction

Figure 3 shows the effect of immobilization on the optimum reaction pH of PEL in *n*-hexane. It was found that the optimum reaction pH of immobilized PEL was 9.5 and that the activity of immobilized PEL decreased greatly when the pH was higher than 10.0 or lower than 8.0. This relation between pH and activity of immobilized PEL was in accord with that of free PEL, indicating that the pH status of free PEL before immobilization could be "memorized" by immobilized enzyme, as observed by Costantino et al. *(20)* and Yang et al. *(21)*. The difference between free PEL and immobilized PEL was that immobilized PEL exhibited obvious activity in a wider pH range.

The effect of immobilization on the optimum reaction pH of enzyme in nonaqueous media mainly depended on the property of the support and the protein charge statement of the enzyme *(22)*. There was a difference in H^+ concentration between the microenvironment of the enzyme and bulk solution resulting from the hydroxy group on the surface of modified USY molecular sieve, which improved the acid tolerance of immobilized PEL. Moreover, the suitable reaction pH value was related to the pore size of the support *(23)*. If the size of the pores is too small, enzyme will not be able to enter the pores of the support as large biomolecules to be immobilized on the surface, and there will be no difference in H^+ concentration between the

Table 2
Impact of Immobilization on Effects of Ions in Resolution of (*R, S*)-2-Octanol[a]

Ion	Concentration (10^{-3} mol/L)	Free PEL activity (%)	Immobilized PEL activity (%)
Control[b]	0	100	100
Ca^{2+}	1	124.12	145.76
	10	150.03	128.11
Mg^{2+}	1	115.88	125.07
	10	123.65	136.35
Na^{+}	1	107.35	110.66
	10	112.09	123.08
K^{+}	1	98.21	111.11
	10	106.18	112.59
Zn^{2+}	1	92.64	125.42
	10	88.52	116.80
Mn^{2+}	1	86.23	123.12
	10	75.39	117.97
Cu^{2+}	1	60.51	126.88
	10	52.43	117.47
Fe^{2+}	1	32.22	124.91
	10	25.38	119.67
Hg^{2+}	1	12.07	107.04
	10	0	92.53

[a]PEL was immobilized onto modified USY molecular sieve and all reactions were carried out at 50°C for 24 h.
[b]No ion added.

microenvironment of the enzyme and the bulk solution; thus, there was little change in the optimum reaction pH. On the other hand, if the size of the pores is too large, the retardant effect on H^+ will be eliminated in the pores of the support and there will also be no difference in H^+ concentration between the microenvironment of the enzyme and the bulk solution; this also led to little change in the optimum reaction pH. In our study, as shown in Fig. 3, the immobilized PEL exhibited obvious activity at a wider pH, although the curve of pH value vs relative conversion rate of immobilized PEL was similar to that of the free PEL. This was mainly because the size of the pores of modified USY molecular sieve was close to that of PEL, so the effects of positional hindrance and diffusion on the reaction pH were reduced to some extent, which improved the acid and alkali tolerance.

Effect of Ions

Table 2 shows the impact of immobilization on the effects of ions during the resolution of (*R, S*)-2-octanol in *n*-hexane. As can be seen, most ions stimulated the activity of PEL immobilized on modified USY molecular sieve to some extent. It was observed that ions such as Fe^{3+}, Cu^{2+}, and Mn^{2+}, which had an inhibitory effect on the free PEL, were enhancers of

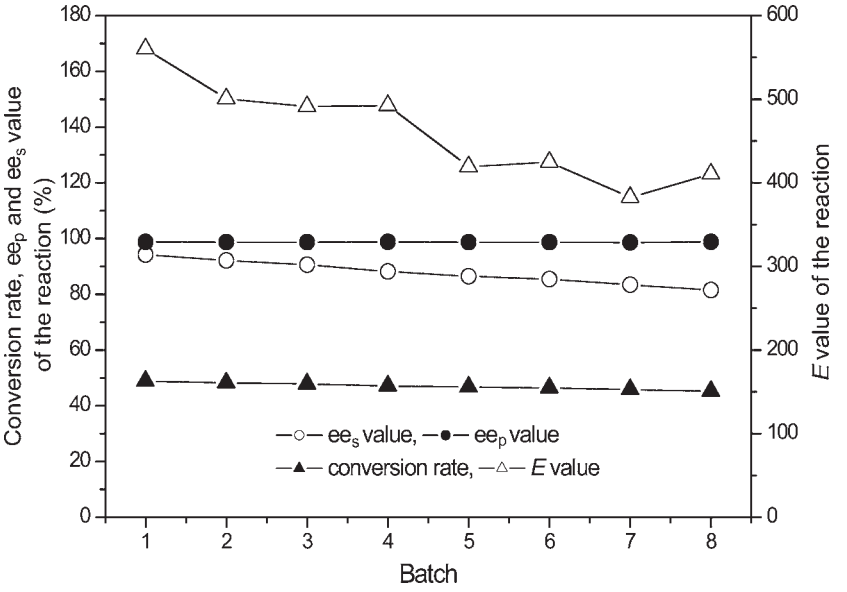

Fig. 4. Resolution of 2-octanol in repeated batch process by immobilized PEL. The immobilized PEL was reclaimed and reused for the enzymatic resolution and reactions were carried out at 50°C for 24 h. Conversion rate, ee_s, ee_p, and *E* value were determined by HPLC.

activity for the immobilized PEL. In addition, Hg^{2+}, an effective inhibitor for free PEL, was an enhancer for immobilized PEL at low concentration but an inhibitor at high concentration, indicating that the process of immobilization of PEL onto modified USY molecular sieve was not a simple process of physical adsorption at equilibrium. There might be an intricate interaction among the support used for immobilization, the microenvironment of PEL, substrates, and the products in nonaqueous media. Further study is needed to clarify the mechanism of the impact of immobilization using modified USY molecular sieve as support on the effects of the ions and whether there are synergistic effects between these ions.

Resolution of (R, S)-2-Octanol in Repeated Batch Process by PEL Immobilized on Modified USY Molecular Sieve

Resolution was performed in a bioreactor in repeated batch process to investigate the reliability of PEL immobilized on modified USY molecular sieve (Fig. 4). The results showed that the average conversion rate of eight batches in succession reached 47% with excellent enantioselectivity (average *E* value of eight batches >460), which was much higher than that reported in the literature for the resolution of (*R, S*)-2-octanol *(1–3)*. This may mainly be owing to the modified USY molecular sieve used for lipase immobilization in addition to the high activity and good enantioselectivity of PEL. As shown in Fig. 4, PEL immobilized on modified USY molecular

sieve was a reliable immobilized lipase, demonstrating a good application potential in the production of optically pure 2-octanol.

Although some chemical methods have been established to prepare racemic compound *(24–26)*, the complicated procedure and the expensive chiral organic reagents required result in a very high cost for industrial application. Because of the substrate specificity and enantioselectivity of enzyme-catalyzed reactions, utilization of enzymes or microorganisms is of great interest in the preparation of optically active chemicals *(27–30)*. The results of our research demonstrated that the immobilization of PEL using modified USY molecular sieve as support was an effective method. The immobilized PEL was a good biocatalyst with fine enantioselectivity in the enzymatic resolution of (*R, S*)-2-octanol.

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